Molecular Insights of Pathways Resulting From Two Common PIK3CA Mutations in Breast Cancer

Poornima Bhat-Nakshatri[1], Chirayu P Goswami[2,§], Sunil Badve[3], Luca Magnani[4], Mathieu Lupien[5], and Harikrishna Nakshatri[1,6,7,#]

[1]Departments of Surgery, [2]Center for Computational Biology and Bioinformatics, [3]Pathology and Laboratory Medicine, [6]Biochemistry and Molecular Biology Indiana University School of Medicine, IN, USA

[4]Division of Cancer, Imperial College, London, UK W120NN.

[5]The Princess Margaret Cancer Centre, University Health Network; Ontario Institute for Cancer Research and Department of Medical Biophysics, University of Toronto, Ontario, M5G 1L7, Canada.

[7]VA Roudebush Medical Center, Indianapolis, IN, USA

Running title: Role of AKT isoforms in estradiol response

Key words: *PIK3CA* mutation, AKT1, AKT2, ERα, estradiol, PI3K inhibitors, breast cancer

#Corresponding author: Harikrishna Nakshatri, C218C, 980 West Walnut St, Indianapolis, IN 46202, USA; Phone 317-278-2238; Fax 317 274 0396; e-mail hnakshat@iupui.edu

§Current Address: Thomas Jefferson University Hospital, Philadelphia, PA 19107, USA

Authors have no conflict of interest to declare

Financial Support: Susan G. Komen for the Cure SAC110025 to H. Nakshatri

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Number of words: 6000

Number of Figures: 6

Number of Tables: 1

List of Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog 1; AP2γ, transcription factor AP2 gamma; E2, estradiol; ER, estrogen receptor, FOXA1, Forkhead box A1, GATA3, GATA binding protein 3; HMEC, human mammary epithelial cells; MEM, minimum essential media; mTOR, mechanistic target of rapamycin; PBX1, pre B cell leukemia homeobox 1; PI3K, phosphatidyl-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homolog; TNBC, Triple Negative Breast Cancer

Abstract:

The phosphatidylinositol 3-kinase (PI3K) pathway is activated in ~70% of breast cancers. PIK3CA gene mutations or amplifications that affect the PI3K p110α subunit account for activation of this pathway in 20-40% of cases, particularly in estrogen-receptor alpha (ERα)-positive breast cancers. AKT family of kinases, AKT1-3, are the downstream targets of PI3K and these kinases activate ERα. Although several inhibitors of PI3K have been developed, none has proven effective in the clinic, partly due to an incomplete understanding of the selective routing of PI3K signaling to specific AKT isoforms. Accordingly, we investigated in this study the contribution of specific AKT isoforms in connecting PI3K activation to ERα signaling, and we also assessed the utility of using the components of PI3K-AKT isoform-ERα signaling axis as predictive biomarkers of response to PI3K inhibitors. Using a variety of physiologically relevant model systems with defined natural or knock-in PIK3CA mutations and/or PI3K hyperactivation, we show that PIK3CA-E545K mutations (found in ~20% of PIK3CA-mutant breast cancers), but not PIK3CA-H1047R mutations (found in 55% of PIK3CA-mutant breast cancers), preferentially activate AKT1. Our findings argue that AKT1 signaling is needed to respond to estrogen and PI3K inhibitors in breast cancer cells with PIK3CA-E545K mutation, but not in breast cancer cells with other PIK3CA mutations. This study offers evidence that personalizing treatment of ER-positive breast cancers to PI3K inhibitor therapy may benefit from an analysis of PIK3CA-E545K-AKT1-estrogen signaling pathways.

Introduction:

The phosphoinositide 3-kinase (PI3K) pathway is a commonly mutated/amplified pathway in cancers (1). Activating mutations of the p110α, the catalytic subunit of PI3K, are common in Estrogen Receptor alpha (ERα)-positive luminal breast cancers, whereas p110α is amplified frequently in ERα-negative basal-like breast cancers (2). These observations suggest a crosstalk between signaling networks emanating from mutant *PIK3CA* and ERα, which impacts breast cancer initiation and/or progression.

ERα is a nuclear receptor activated in response to its ligand estradiol (E2), and plays a significant role in >70% of breast cancers (3,4). In addition to E2, multiple co-factors and posttranslational modifications control ERα activity (4,5). These include pioneer factors such as FOXA1, GATA3, and AP2γ that guide ERα binding to the genome, transcriptional co-regulators that influence transcriptional output from ERα, and ERα phosphorylation that influences transcriptional activity, stability, and subcellular distribution (4). Several growth factor receptor activated kinases including AKT intersect with ERα signaling by phosphorylating ERα and altering transcriptional output (6).

AKT family of kinases (AKT1-3) are frequently activated downstream of PI3K. Published work from multiple groups including ours has shown significant influence of AKT in ERα phosphorylation, genome-wide binding, E2-dependent mRNA and microRNA expression, and alternative splicing (7-11). We demonstrated distinct prognostic value of nuclear phospho-AKT in ERα-positive breast cancers (12). However, there are two significant gaps in our understanding of crosstalk between PI3K and ERα signaling, which this study is designed to address. First, the isoform of AKT that preferentially engages PI3K with ERα is unknown. Second, it is unknown whether

PIK3CA-E545K mutation, which represents 20% of *PIK3CA* mutations, and *PIK3CA-H1047R* mutation, which represents 55% of PIK3CA mutations in breast cancer (mycancergenome.org), has similar influence on AKT isoform activation. Exploring these gaps is critical because of recent understanding that AKT isoforms are not functionally similar (13,14). Moreover, commonly used constitutively active AKT mutants do not discriminate the functions of different isoforms of AKT (13). We focused on AKT1 and AKT2 because AKT3 is relevant for only ERα-negative breast cancers (15,16). Our results showed that, in general, *PIK3CA-E545K* mutation is associated with AKT1 activation, whereas *PI3KCA-H1047R* mutation with activation of AKT1, AKT2, or both. AKT1 is essential for ERα activity, E2-dependency, and response to PI3K inhibitors in MCF-7 cells with endogenous *PIK3CA-E545K* mutation. Thus, response of ERα-positive breast cancers to PI3K inhibitors may depend on the isoform of AKT activated as a consequence of specific *PIK3CA* mutation.

Materials and Methods:

Cell lines: MCF-7, T47-D, LY2, HCC1428, BT-474, SK-BR-3, MDA-MB-231, MDA-MB-436, MDA-MB-468, UACC812, and ZR-75-1 cells were purchased from ATCC. 600MPE cell line was a gift from Dr. Paul Spellman (17). HCC1428, UACC812 and LY2 cell lines were purchased within last one year and other cell lines have been authenticated within past two years using STR Systems for Cell line identification (DNA Diagnosis Center, Fairfield, OH and Genetica DNA Laboratories, Cincinnati, OH). Human immortalized mammary epithelial cells (HMECs) with targeted replacement of *PIK3CA* with *PIK3CA-E545K* and *PIK3CA-H1047R* were purchased from Horizon Discovery Limited (hTERT-HME1, Cambridge, UK). Drs. Ben Ho Park and Michele Vitolo provided MCF10A cells with targeted *PIK3CA* mutants and *PTEN* deletion, respectively (18,19). Dr. Alex Toker provided the parental pLKO, AKT1, and AKT2 shRNA lentivirus vectors (20). Supplementary information has additional details of lentivirus transfection, siRNAs, and cell proliferation assays.

Antibodies: Antibodies against AKT1, AKT2, AKT1_pS473, AKT2_pS474, and phospho-GSK3α/β (S9/21) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ERα, GATA3, GSK3α/β, FOXA1, cMyc, and Cyclin D1 were purchased from Santa Cruz Biotechnology, whereas AP2γ antibody was from Epitomics (Burlingame, CA).

RNA isolation, Microarray, and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR): RNA was prepared using RNeasy kit (Qiagen, Valencia, CA) and

cDNA from two µgs of RNA was synthesized using the cDNA Synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using SyberGreen on an Applied Biosystems 7900HT instrument (Applied Biosystems, Carlsbad, CA). Sequences of primers used for qRT-PCR are in Supplementary Table S1. Microarray with biological triplicates was performed using Illumina HumanHT-12 V4 expression beadchip. Supplementary information contains additional details of microarray data analyses and results have been submitted to Gene Expression Omnibus (GSE60759).

Transcription factor binding site (TFBS) enrichment analyses: The TFBS enrichment analysis was done using the MotifModeler tool (21). Briefly, MotifModeler uses array based gene expression data to identify functional binding sites by combining effects of binding sites of different transcription factors and estimating functional effects of predicted motifs under contrasting conditions. The tool makes assumption that differential gene expression is a feature of altered regulation and deduces differentially acting regulatory elements from a dataset of all regulatory elements based upon a list of differentially expressed genes. These binding sites are located within 1000 bases 5' of the transcription start site and in the annotated 3' untranslated region of genes. ERα ChIP-on-chip data and ChIP-seq data from our previous studies were used to assign ERα binding sites (8,22).

Analysis of signaling pathways overlap: FOXA1-E2 and PBX1-E2 signatures have been described previously (23). Gene lists for AKT1 and AKT2 dependent E2-induced genes were imported into Oncomine (24). Overlap was defined as significant at *p* value of at

least 0.01 and an Odds Ratio 2. Prognostic impact of FOXA1-E2-ERα-AKT1 signature was evaluated using two public databases (25,26).

Results:

AKT isoform activity in breast cancer cell lines with endogenous *PIK3CA* aberrations.

Recently developed antibodies that recognize activated AKT1 (AKT1_pS473) and AKT2 (AKT2_pS474) enabled us to reexamine whether specific isoforms are activated in response to distinct PI3K aberrations. Based on the studies using pan AKT_pS473 antibody, it was suggested that *PIK3CA* mutation is not always associated with AKT activation. For example, MCF-7 cells with *PIK3CA-E545K* mutation were reported to lack constitutive AKT activity (27). In contrast to the results reported using pan AKT_pS473 antibody, AKT1_pS473 was readily detected in MCF-7 cells under serum-deprived and serum-supplemented conditions (Figure 1A). AKT1_pS473 and AKT2_pS474 levels (to a lesser extent) in MCF-7 cells under serum-treated condition were higher than in 600MPE cell line, which lacks *PIK3CA/PTEN* alterations. AKT2_pS474 was dominant in T47-D cells with *PIK3CA-H1047R* mutation as its levels were higher compared with MCF-7 or 600MPE cell lines. Several additional cell lines (12 cell lines; LY2 is an anti-estrogen resistant derivative of MCF-7) were examined to determine whether *PIK3CA* mutation, *PTEN* mutation and/or *HER2* amplification correlates with elevated basal and serum-inducible activated AKT1 or AKT2. All *HER2*-amplified cell lines (BT-474, SK-BR-3, and UACC812) displayed elevated basal AKT1_pS473, AKT2_pS474, or both. For example, UACC812 cells with both *HER2* amplification and *PIK3CA-N345K* mutation displayed elevated AKT2_pS474, whereas SK-BR-3 with *HER2* amplification showed elevated AKT1_pS473 compared with LY2 cells (Figure 1A). BT-474 cells with *HER2* amplification and *PIK3CA-K111N* mutation

displayed elevated levels of both AKT1_pS473 and AKT2_pS474 compared with 600MPE or MCF-7 cells. Although additional well-characterized cell lines need to be examined, there was a trend of elevated AKT2_pS474 in cell lines with EGFR family amplification or overexpression (BT-474, UACC812, MD-468, and Hs578t). We also noted cell type-specific differences in serum-inducible AKT1 and AKT2 activity, as serum did not affect the levels of AKT1_pS473 and AKT2_pS474 in BT-474, ZR-75, LY2, UACC812, and MD-468 but increased their levels in MCF-7, T47-D, SK-BR-3, and Hs578t cells. Thus, multiple genomic events including *PIK3CA* mutation control AKT1 and AKT2 activity.

AKT isoform activity in immortalized breast epithelial cell lines with *PIK3CA-E545K* or *PIK3CA-H1047R* mutation. Since *PIK3CA-E545K* and *PIK3CA-H1047R* are the most common PIK3CA mutations in breast cancers, we further defined the link between specific PIK3CA mutation and AKT isoform activity using knock-in cell lines. HMECs and MCF10A cells with targeted replacement of one *PIK3CA* allele with either *E545K* or *H1047R* mutant allele were used (18). MCF10A cells with homozygous deletion of *PTEN* were used as a positive control (19). Under serum-starved condition (24 hours), wild type HMECs displayed residual AKT1 activity, which was significantly higher in E545K and H1047R cells (Figure 1B). Under serum and growth factor supplemented condition, mutant cell lines displayed elevated AKT1 activity. Although *PIK3CA* mutation increased AKT2 activity, the effects were relatively modest. Thus, AKT1 is the major target of *PIK3CA* mutation in HMEC cells. Increased AKT1 activity

in HMEC-E545K and HMEC-H1047R mutant cells correlated with elevated levels of its substrate phospho-GSK3α/β (Figure 1C).

Results were markedly different in MCF10A derivatives. Basal AKT1_pS473 and AKT2_pS474 levels were similar in serum-deprived parental and mutant MCF10A cells (Figure 1D). *PTEN* deletion and *PIK3CA-H1047R* mutation but not *PIK3CA-E545K* mutation resulted in elevated heregulin-induced AKT1_pS473 and AKT2_pS474 levels. These results indicated that *PIK3CA* mutations have cell type-specific effects on AKT1_pS473 and AKT2_pS474 levels under basal and/or growth factor stimulated condition. Interestingly, two out of three cell lines with *PIK3CA-E545K* mutation (HMEC-E545K and MCF-7 but not MCF10A-E545K) but no known growth factor receptor amplification, PTEN loss or K-Ras mutation showed preferential elevation of AKT1_pS473. By contrast, all *PIK3CA-H1047R* cell lines without growth factor receptor amplification, PTEN loss or K-Ras mutation (HMEC-H1047R, MCF10A-H1047R and T47-D) showed elevated levels of AKT1_pS473, AKT2_pS474, or both. Thus, it appears that, in the absence of other known confounding genomic events, *PIK3CA-H1047R* mutation is more potent in activating AKT than *PIK3CA-E545K* mutation. These results reveal distinct AKT isoform-driven signaling/biology downstream of *PIK3CA-E545K* and *PIK3CA-H1047R* mutation, which, to our knowledge, is not routinely considered while assessing the impact of *PIK3CA* aberration on cancer progression.

Deciphering PIK3CA-E545K:AKT isoform:ERα signaling axis in MCF-7 cells.

One of our focuses was to determine how *PIK3CA* mutation and specific AKT isoform activation influence ERα activity. MCF-7 cells are ideal for this purpose because these cells are ERα-positive, E2-dependent, and contain *PIK3CA-E545K* mutation. A

recent study demonstrated that *PIK3CA-E545K* mutation is responsible for AKT activity in MCF-7 cells (28). MCF10A, HMECs and their PIK3CA mutant derivatives are not ideal because these cells do not express ERα and E2 treatment did not alter phosphorylation status of AKT1 or AKT2 in wild type or mutant MCF10A cells (data not shown). Since we observed an association between *PIK3CA-E545K* mutation and AKT1 activation in MCF-7, our goal was to further evaluate whether AKT1 integrates signaling from *PIK3CA-E545K* mutation to ERα through AKT1 or AKT2. Towards this end, we generated cells expressing shRNA against AKT1 or AKT2 using previously validated shRNA constructs (20) (Figure 2A). Interestingly, AKT1 knockdown in these cells resulted in compensatory increase in AKT2.

We first examined pAKT_pS473 levels in MCF-7pLKO, AKT1KD, and AKT2KD cells upon activation of PI3K by heregulin. Heregulin-mediated AKT activation was lower in AKT1KD cells compared with control pLKO or AKT2KD cells (Figure 2B). There are two possible explanations for the observed effects of AKT1 knockdown on heregulin-induced pAKT_pS473 levels; one is that heregulin preferentially activates AKT1 and the second is that AKT_pS473 antibody preferentially recognizes phosphorylated AKT1. To test these possibilities, we probed untreated and heregulin-treated MCF-7pLKO, AKT1KD and AKT2KD cells with AKT1_pS473 and AKT2_pS474 antibodies. As expected, heregulin-induced AKT1_pS473 levels were lower in AKT1KD cells (Figure 2C). Heregulin-induced AKT2 activation was unaffected in AKT1KD but reduced in AKT2KD cells. Therefore, lower levels of AKT_pS473 in heregulin-treated AKT1KD cells compared with pLKO or AKT2KD cells could be due to preferential recognition of phosphorylated AKT1 by this antibody. We consistently

observed lower AKT1_pS473 levels in AKT2 knockdown cells suggesting a regulatory role for AKT2 in controlling AKT1 activity. Similar results were obtained when cells were stimulated with insulin; AKT1_pS473 levels were lower in insulin-treated AKT1KD and AKT2KD cells compared with pLKO cells (Figure 2D).

We performed two sets of experiments to link specific AKT isoforms to E2:ERα signaling. First, we examined whether AKT1 and AKT2 isoforms control the expression of ERα and pioneer factors that regulate ERα (29). AKT1 or AKT2 knockdown did not alter ERα, GATA3, and FOXA1 levels. Basal expression of AP2γ was lower in AKT1KD compared with pLKO or AKT2KD cells (Figure 3A). Among E2 inducible proteins tested, AKT1 or AKT2 knockdown had minimal effect on cMyc induction, whereas knockdown of either isoforms reduced E2-inducible Cyclin D1.

Second, we performed microarray analysis of untreated and 3-hours E2-treated cells. AKT2 knockdown had greater effect than AKT1 knockdown on basal expression of genes (2982 genes versus 893 genes out of 15704 genes at *p*-value of <0.01 in the array with measurable expression, p=0.0001, Chi-square with Yates correction) (Table 1). In fact, AKT2 knockdown increased the basal expression of 2955 genes, which was significantly higher than the effect of AKT1 knockdown (656 genes, p=0.0001 AKT1 vs. AKT2). Thus, AKT2 potentially serves as a global suppressor of gene expression. Alternatively, distinct role of AKT1 and AKT2 in cell cycle may have indirectly contributed to differences in basal gene expression between three cell types (30). Names and fold-change in expression of genes along with ERα binding pattern to these genes, determined based on previously described ChIP-seq or ChIP-on-Chip datasets, are presented in Table S2. TFBS enrichment analysis of genes differentially expressed under

basal condition revealed potential interaction between specific transcription factors and AKT isoform activated signals. For example, binding sites for TEF1, ATF6, AP2rep, SREBP1, ZF5, and CREB were observed in genes that are differentially expressed in AKT1KD or AKT2KD cells compared with pLKO cells (Figure 3B). By contrast, genes differentially expressed in AKT1KD cells were enriched for E2F, AP1, SP1, myogenin, AP2γ, AP2α, STAT6, NERF1a, CP2, and NRF2 binding sites. Genes differentially expressed in AKT2KD cells were enriched for LEF1, AML, PEA3, SRY, MAZ, CRX, and USF2. Thus, AKT1 and AKT2 may target different transcription factors to modulate basal expression of genes.

With $p<0.01$, we found 1912 genes to be E2-regulated in pLKO cells with 1682 genes being induced. When the expression of these E2-regulated genes was analyzed after normalizing for basal expression in AKTKD cells, AKT1KD demonstrated higher effect than AKT2KD on E2-regulated gene expression (Table 1 and Table S3). For example, AKT1KD decreased E2-regulated expression of 405 genes but increased E2-regulated expression of 33 genes. By contrast, AKT2 knockdown resulted in decreased E2-regulated expression of 201 genes but increased the expression of 15 genes. Only 104 E2-regulated genes were commonly affected upon AKT1 or AKT2 knockdown. The differences in number of E2-regulated genes between AKT1KD and AKT2KD cells are statistically significant ($p=0.0001$, Fisher's exact test, two-tailed). In summation, while AKT1 significantly influenced E2-regulated gene expression, AKT2 displayed a global role in controlling basal gene expression.

We subjected E2-regulated genes differentially affected by AKT1 or AKT2 to Ingenuity Pathway analysis. E2-regulated genes requiring AKT1 are involved in

engaging ERα to NF-κB, p53-CDKN2A, and PI3 kinase-AKT-MAPK8-ERK pathways (Figure S1). By contrast, E2-regulated genes requiring AKT2 are involved in p53-ERBB2-CCND1, FOS-Myc-C/EBPβ, and RNA polymerase II-Histone H3-ERK1/2 pathways (Figure S2). Pathway analysis revealing a link between AKT1 dependent E2-regulated genes and PI3K pathway further suggests the involvement of AKT1 in integrating PI3K activation to ERα signaling.

AKT1-E2 and AKT2-E2-dependent genes are enriched for unique transcription factor binding sites.

To obtain insight into how AKT isoforms might differentially control the expression of E2 regulated genes, we assigned binding sites for ERα, FOXA1, GATA3, p300, CBP, SRC1, SRC2, and SRC3 to each of the E2-regulated genes that were differentially affected by AKT isoforms (Table S3). Binding sites of these transcription co-regulators within 10-kB upstream of transcription start site and 5-kB downstream of the 3' end of E2-regulated genes in MCF-7 cells have been described (31-33). Less than 5% of genes differentially expressed in both cell types contained binding sites for p300, CBP, or any of the SRCs (Table S3). 50%, 60%, and 80% of differentially expressed genes contained ER, FOXA1, and GATA3 binding sites, respectively, with no AKT-isoform-specific enrichment of binding sites.

Binding sites for the pioneer factors GATA3 and PBX1 were enriched in genes whose E2-dependent expression was affected in both AKT1KD and AKT2KD cells (Figure 3C). AP2γ, STAT5, and FOXO1 binding sites were enriched in genes that were dependent on AKT1 for E2-regulated expression (Figure 3C). By contrast, binding sites

for NF-κB and NKX2-5 were enriched in genes that were dependent on AKT2 (Figure 3C).

It is interesting that few of the transcription factors noted above have previously been shown to be E2-regulated and mediate secondary E2 response (34). AKT isoform dependency of several of the E2-regulated genes could be due to differential influence of AKT1 and AKT2 isoforms on E2-dependent expression of these transcription factors. Indeed, majority of these transcription factors including E2F family members are E2-regulated in our cell lines and AKT1 and AKT2 knockdown had differential effect on their E2-regulated expression (Figure 3D). Collectively, these results suggest that AKT isoforms differentially regulate primary and secondary E2-response genes.

Cyclin D1, in addition to being E2-inducible, interacts with ERα and influences the expression of >2000 E2-regulated genes (35). We analyzed the effect of AKT isoform knockdown on top 50 of Cyclin D1-ERα-regulated genes. Out of 38 genes that overlapped between Cyclin D1-ERα and AKT-isoform-ERα datasets, E2-regulated expression of only two genes intersected with Cyclin D1-ERα and AKT1-ERα axis (MSMB and IL17RB, Table S3) and none with AKT2. Thus, AKT isoforms appears to have minimum role in regulating Cyclin D1-ERα axis.

Identification of PIK3CA-E545K:AKT1:ERα:E2 gene expression signature.

Among the pioneer factors that control ERα:E2-mediated gene expression, prognostic utility of E2:ERα:FOXA1 and E2:ERα:PBX1 regulated genes has been described (23). FOXA1-E2 regulated genes are associated with good outcome whereas PBX1-E2 regulated genes are associated with poor outcome. We restricted our analysis to genes of these signatures and determined the influence of AKT1 and AKT2 on their

expression. Neither AKT1 nor AKT2 had an influence on PBX1-E2 gene expression signature. By contrast, FOXA1-E2 dependent genes were preferentially associated with AKT1 ($p<7.9\ 10^{-9}$ and Odd ratio 17.7) (Table S4).

To determine the influence of E2-regulated genes that are additionally controlled by AKT1 (called FOXA1-E2-AKT1 signature hereafter), we analyzed the impact of FOXA1-E2-AKT1 signature in the publicly available dataset, which allowed a combined analysis of tumors of 2977 patients (25). Data for CXCL12, PLAC1, SGK1, SLC22A5, and TGM2 were available in this database and all of them were positively regulated by AKT1. Elevated expression of genes in the signature was associated with better recurrence-free survival in luminal A and luminal B breast cancer (Figure 3E). This signature had similar effect on outcome in patients treated with endocrine therapy, tamoxifen therapy, or surgery alone (Figure 3E). Also, the signature predicted better distant metastasis-free survival in patients with luminal A, luminal B breast cancers, and patients who did not receive systemic therapy (Figure 3E). Thus, FOXA1-E2-AKT1 signature likely defines a cancer type with robust hormone receptor activity.

We independently validated the above results using a resource developed by our group (26). In Wang et al dataset (36), elevated expression of this signature was associated with favorable recurrence-free survival among patients with ERα-positive breast cancer (Figure S3). In the Loi et al dataset (37), elevated expression of this signature was associated with better outcome in tamoxifen-treated patients. In the TCGA dataset, elevated expression of this signature was associated with favorable overall survival of patients with PR-positive and a trend in ERα-positive breast cancer.

We performed additional analysis to find a relationship between genes differentially influenced by AKT1 or AKT2 under basal or E2-treated condition and various other ERα-positive breast cancer-specific prognostic signatures including SRC-1, SRC-2, SRC-3, p300, CBP (32), and tamoxifen resistance signatures (38). AKT1 or AKT2 knockdown had minimum effect on the expression of genes in these signatures. Thus, the signature identified above is unique to AKT1 and is not related to other signatures of ERα-positive breast cancer.

Recent studies have described an ERα-dependent E2F-mediated resistance to aromatase inhibitors (39). Our array contained detectable expression of 17 out of the 24 genes of this signature. Nine of these genes at $p<0.01$ were E2 inducible (Table S4). Seven of these genes lost E2 inducible expression in AKT1KD or AKT2KD cells. Thus, AKT influences the expression of genes associated with anti-estrogen resistance, although the effects are not isoform-specific.

Validation of E2-regulated genes that are differentially influenced by AKT1 and AKT2 in MCF-7 cells.

We performed qRT-PCR analysis of several of the genes in the FOXA1-E2-AKT1 signature and other E2-regulated genes to verify the effects of AKT1 and AKT2 knockdown. Consistent with the results of microarray, E2 failed to increase RERG and KCNK6 in AKT1KD cells compared with parental cells (Figure 4A). Although RERG expression was partially affected in AKT2KD cells, KCNK6 was E2-inducible in these cells. Similarly, E2-inducible expression of SIAH2 was significantly lower in AKT1KD cells compared with pLKO or AKT2KD cells. E2-inducible expression of SLC22A5 was lower in AKT1KD cells compared with AKT2KD cells. With respect to E2 repressed

genes, E2 readily repressed embryonic stem cell gene SALL4 in pLKO cells (40), which was inefficient in AKT1KD cells (Figure 4B). E2-mediated repression of BTG2, a breast cancer tumor suppressor (41), was enhanced in AKT1KD and AKT2KD cells (Figure 4B). These results further confirm the ability of AKT1 and AKT2 to distinctly modulate E2-regulated gene expression.

AKT1 influences response of MCF-7 cells to E2 and PI3K inhibitors.

We next examined the effect of AKT1 and AKT2 knockdown on E2-stimulated proliferation. Early passage AKT1KD cells demonstrated least E2-stimulated proliferation (Figure 5A). However, AKT knockdown cells remained as sensitive as pLKO cells to tamoxifen (Figure 5B).

We next tested whether AKT1 isoforms have specific role in determining sensitivity to PI3Kα-specific inhibitor BYL719, pan-PI3K inhibitor BKM120, PI3K/mTOR dual inhibitor NVP-BEZ235, and pan-AKT inhibitor MK2206. AKT1 levels determined sensitivity to BYL719, as AKT1KD cells were resistant to this drug compared with pLKO or AKT2KD cells (for example, 20% survival of pLKO cells compared with 80% survival of AKT1KD cells when treated with 100 nM drug, Figure 5B). Interestingly, E2 treatment significantly reduced sensitivity to this drug irrespective of AKT isoform knockdown suggesting the existence of E2:ERα-dependent resistance mechanism to this class of inhibitors. AKT1KD cells demonstrated a modest increase in sensitivity to BKM120 under basal growth condition but the sensitivity was reversed upon E2 addition (Figure 5C). AKT1 and AKT2 knockdown had statistically significant effects on sensitivity to NVP-BEZ235 and MK2206 but the effects were numerically modest (Figure 5D and E). AKT2KD cells showed resistance to MK2206 under E2

treated condition (Figure 5E). These results indicate that AKT1 has significant influence on sensitivity to PI3K-specific inhibitors compared with AKT2.

AKT isoforms are not essential for E2 response in BT-474 cells.

To determine whether AKT1:ERα signaling axis extends beyond MCF-7 cells, we examined the role of AKT1 and AKT2 in E2 and PI3K inhibitor response in BT-474 with *PIK3CA-K111N* mutation and *HER2* amplification. In these cells, knockdown of both AKT1 and AKT2 reduced heregulin-induced AKT1_pS473 levels (Figure 6A). By contrast, unlike in MCF-7 cells, AKT1 knockdown reduced heregulin-induced AKT2_pS474 levels. Thus, type of crosstalk between AKT isoforms is cell type-specific.

Unlike in MCF-7 cells, where AKT1 and AKT2 knockdown did not reproducibly affect basal proliferation (data not shown), knockdown of both isoforms of AKT reduced basal proliferation but not E2-inducible proliferation of BT-474 cells (Figure 6B and C). Consistent with the limited role of AKT isoforms in E2-mediated proliferation, E2-induced SIAH2 expression was unaffected by either AKT1 or AKT2 knockdown in these cells (Figure 6D). With respect to PIK3CA/mTOR inhibitor response, BT-474 cells were not sensitive to BYL719 (data not shown). However, these cells were sensitive to NVP-BEZ235 (Figure 6E). Interestingly, AKT1KD and AKT2KD cells showed partial resistance to this drug, although statistical significance was achieved only in AKT2KD cells. Thus, AKT inhibition may force BT-474 cells to adapt to alternative survival mechanism independent of PI3K signaling.

Discussion

Discovery of recurrent activating mutation of *PIK3CA* in a variety of cancers has prompted the development of PI3K inhibitors as cancer therapeutics (42,43). Response to these inhibitors has not always been correlated with *PIK3CA* mutations suggesting that additional pathways control therapeutic response. In this regard, Myc, eIF4E, RSK3/4 and JAK/STAT pathways are suggested to be involved in conferring resistance to these inhibitors (1). Our results show significant cell type specificity in AKT isoform activation upon *PIK3CA* mutation and the unique ability of activated AKT1 downstream of *PIK3CA-E545K* mutation in determining E2 response and sensitivity to PI3K inhibitors.

Recently developed antibodies against activated AKT1 and AKT2 allowed us to make major stride in defining the role of AKT isoforms in the context of *PIK3CA* mutation. We observed cell type-specific differences in basal levels of activated AKT1 and AKT2 suggesting that genomic aberrations can lead to biased activation of these isoforms. These reagents allowed us to reexamine one of the earlier observations regarding basal AKT activity in breast cancer cell lines with *PIK3CA* mutation. For example, MCF-7 cells, despite containing *PIK3CA-E545K* mutation, were reported to lack activated AKT based on western blot analysis with AKT_pS473 antibody (27). However, we detected AKT1_pS473 in these cells.

Cell type specific role of AKT isoforms in E2 signaling.

AKT1 knockdown had much higher effect on E2-regulated gene expression and proliferation compared with AKT2 knockdown in MCF-7 cells. AKT1 activation in MCF-7 cells is dependent on *PIK3CA-E545K* mutation (28) and results presented in Figures 1 and 2 suggest preferential effect of this *PIK3CA* mutation on AKT1 activation. Therefore, *PIK3CA* mutation in this cell line could contribute to ERα activity through

activation of AKT1. In this regard, among ERα-positive cell lines, MCF-7 has been the workhorse for ERα-related studies because of its dependence on E2 for survival and studies using this cell line have provided clinically applicable biomarkers of anti-estrogen sensitivity. Unfortunately, there is no other ERα-positive cell line with *PIK3CA-E545K* mutation to verify the results of MCF-7 cells.

In BT-474 cells with distinct *PIK3CA* mutation and *HER2* amplification, AKT isoforms were not required for E2 response (Figure 6). Alternative signaling pathway activation due to *HER2* amplification may have rendered AKT isoforms redundant for ERα signaling in these cells. HER2 can activate MAPKs, which can also increase ERα activity (44). Therefore, multiple genomic aberrations need to be taken into consideration before concluding whether mutant *PIK3CA* integrates with ERα signaling through AKT isoforms.

The mechanisms leading to preferential engagement of AKT1 over AKT2 in eliciting optimal E2 response in MCF-7 cells are unknown. Both AKT1 and AKT2 phosphorylate ERα; therefore, difference in ERα phosphorylation is less likely to account for differential effects on E2 signaling (7,45). AKT1 and AKT2 share common upstream activators but frequently target distinct downstream molecules such as EMSY and Palladin (20,46). Therefore, a likely scenario includes specific engagement of AKT1 with ERα co-regulatory molecules, histone modifying enzymes, or pioneer factors. We observed lower expression of the pioneer factor AP2γ in AKT1KD cells and AP2γ binding sites are enriched in genes that are dependent on AKT1 (Figure 3). A recent study demonstrated a role of AP2γ in chromatin binding of unliganded ERα and unliganded ERα regulating AP2γ expression (47). AP2γ is also essential for ERα-

mediated long-range chromatin interaction (48). It is likely that AKT1 serves as a central node in connecting AP2γ to ERα signaling. Since our results showed specific effects of AKT1 on E2-regulated gene expression and AKT2 on basal gene expression in MCF-7 cells, we propose that, depending on the cell type, some of the genomic effects attributed to PI3K/AKT signaling are dependent on isoforms of AKT that are activated. Within this gene network, FOXA1:ERα:E2:AKT1 activated signature is associated with better clinical outcome, similar to the recently described ERα:E2:PLK1 signature (49). These specific kinase dependent E2 gene expression signatures are likely predictive markers of E2-dependency and response to targeted therapies. In this context, AKT1 knockdown reduced the sensitivity to PI3K inhibitors in MCF-7 cells (Figure 5), suggesting that lower levels of AKT1 force cells to adapt to alternative survival pathways. Alternatively, AKT1 may actively suppress the activation of compensatory survival pathways similar to the previously suggested role of AKT in suppressing Raf-dependent ERK activation (50). Clinically, tumors with active AKT1 and ERα signaling may show higher sensitivity to PI3K inhibitors. However, developing markers of sensitivity to these drugs may prove difficult because of cell type specificity observed in the effects of *PIK3CA* mutation on signaling by AKT isoforms. Nonetheless, focused analyses of PIK3CA-E545K-AKT1-ERα signaling axis may be useful in select number of cases.

Acknowledgements: We thank Drs. Spellman, Ben Ho Park, and Michele Vitolo for 600MPE cell line, MCF10A cells with *PIK3CA* mutation and MCF10A cells with *PTEN* deletion, respectively. We also thank Dr. Alex Toker for AKT shRNA constructs.

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Table 1: The effects of AKT1 and AKT2 knockdown on basal and E2-regulated gene expression. Note that basal expression differences between cell types were normalized to one before calculating the effects of AKT1 and AKT2 knockdown on E2-regulated gene expression.

Basal gene expression

	Up-regulated	Down-regulated
AKT1 shRNA vs. Control	656*	237
AKT2 shRNA vs. control	2955*	27

*p=0.001

E2-regulation after normalization of basal expression.

Number of E2-inducible genes in parental cells	1682
Number of E2-repressed genes	230
Number of E2-regulated genes whose expression is decreased upon AKT1 knockdown	405**
Number of E2-regulated genes whose expression increased upon AKT1 knockdown	33
Number of E2-regulated genes whose expression decreased upon AKT2 knockdown	201**
Number of E2-regulated genes whose expression increased upon AKT2 knockdown	15

**p=0.0001

Figure Legends:

Figure 1: Relationship between *PIK3CA* mutation and AKT isoform expression/activity. A) AKT1_pS473 and AKT2_pS474 levels in breast cancer cell lines with and without *PIK3CA/PTEN* mutation and *HER2* amplification. Data from cells grown in regular media as well as serum starved for 24 hours are shown. B) AKT1 and AKT2 activation in HMECs with targeted replacement of one copy of wild type *PIK3CA* with indicated mutants. Active AKT1 and AKT2 were measured using phospho-specific antibodies. Results from three batches of lysates from serum-starved cells are shown. C) PI3K-dependent activation of AKT1 in HMECs correlates with activation GSK3α/β, its downstream target. D) AKT1 and AKT2 activation in MCF10A cells with targeted replacement of one copy of wild type *PIK3CA* with indicated mutation or deletion of PTEN. PTEN-/- cells and PIK3CA mutant cells were obtained from different labs; therefore, wild type cells from corresponding labs were included as controls.

Figure 2: AKT1 and AKT2 activity in AKT isoform knockdown MCF-7 cells. A) AKT1 and AKT2 levels in AKT1KD and AKT2KD MCF-7 cells. B) Effects of AKT1 and AKT2 knockdown on heregulin-induced AKT phosphorylation. C) The effect of AKT1 and AKT2 knockdown on basal and heregulin inducible (15 minutes treatment) levels of AKT1_pS473 and AKT2_pS474. Longer exposure of the gel is shown to demonstrate the effects of knockdown on basal AKT1 and AKT2 activity levels (*). D) The effect of AKT1 and AKT2 knockdown on insulin-mediated (INS, 50 ng/ml for 15 minutes) AKT1 and AKT2 activation.

Figure 3: The effects of AKT1 and AKT2 knockdown on E2 signaling in MCF-7 cells. A) Expression levels of ERα, pioneer factors, and E2-inducible proteins in different cell types. B) TFBS enriched in genes expressed differentially in AKT1KD and

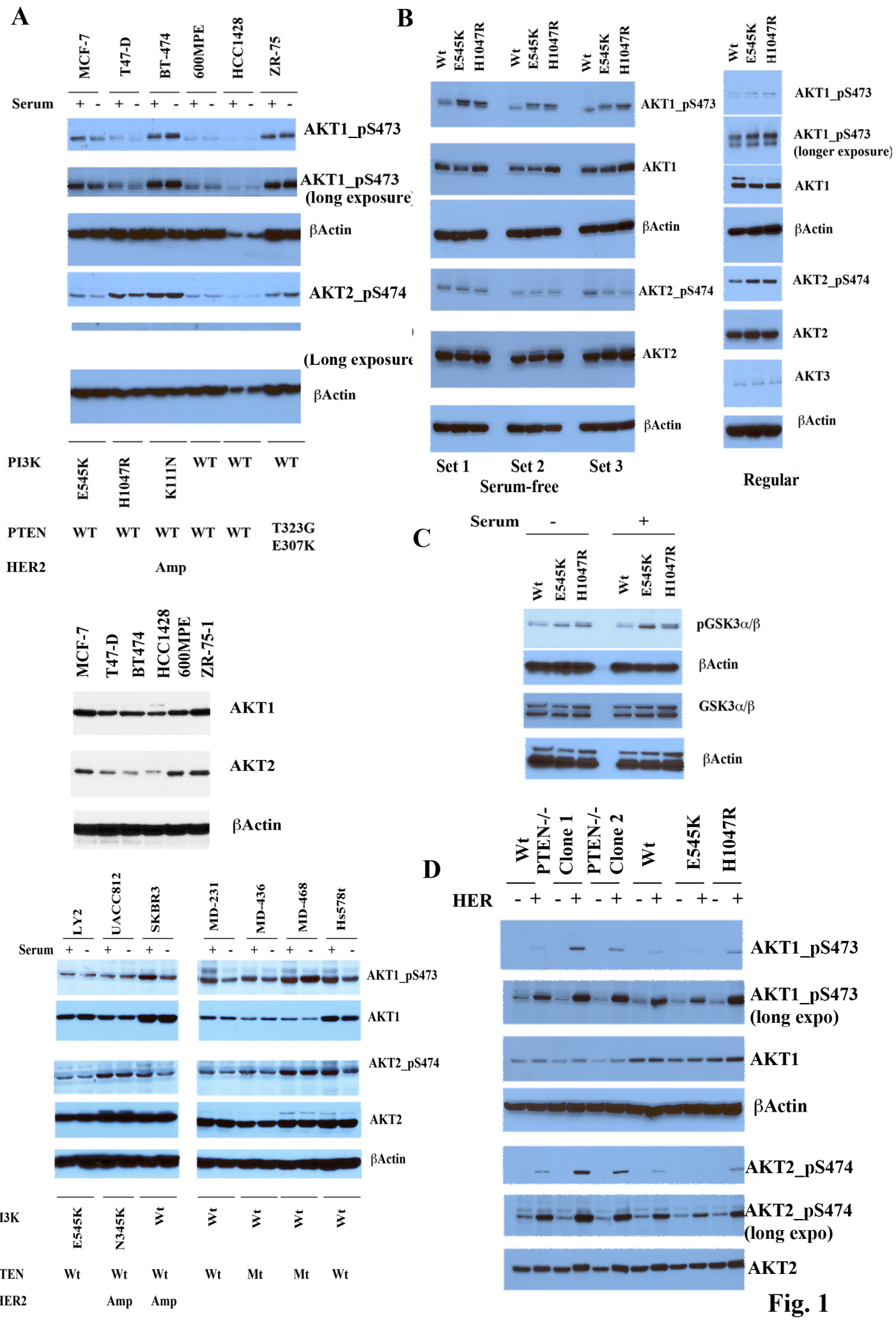
AKT2KD cells compared with pLKO cells under basal condition. C) TFBS enriched in genes expressed differentially in AKT1KD and AKT2KD cells compared with pLKO cells under E2-treated condition. D) The effects of E2, AKT1 and AKT2 knockdown on the expression of transcription factors potentially involved in the expression of E2-regulated genes. Table S2 provides detailed fold changes and p-values. E) Prognostic value of E2:ERα:FOXA1:AKT1 signature. Kaplan-Meier curves for recurrence-free (RFS) and metastasis-free survival (DMFS) of breast cancer subtypes are shown. Gene expression pattern was split at median to classify as high or low expressers.

Figure 4: Validation of the effects of AKT1 and AKT2 knockdown on E2-regulated gene expression. A) AKT1 but not AKT2 knockdown significantly reduced E2-inducible expression of KCNK6, RERG, and SIAH2 in MCF-7 cells. Basal expression in all three-cell types was normalized to one. Mean ± standard errors (SE) are shown. B) AKT1 and AKT2 had gene-specific effects on E2-mediated gene repression.

Figure 5: AKT1 and AKT2 isoform knockdown had distinct effects on E2-induced proliferation and PI3K/mTOR/AKT inhibitor sensitivity of MCF-7 cells. A) The effects of AKT1 and AKT2 knockdown on E2 induced proliferation. Cells were treated with E2 (0.1 nM), 4-hydroxy tamoxifen (Tam, 100 nM) or both for six days and cell proliferation was measured using bromodeoxyuridine-incorporation ELISA. Mean ±SE are shown. B) AKT1 knockdown had significant effect on sensitivity to BYL719. Cells were grown without (left) or with E2 (right). C) AKT1KD cells were more sensitive to BKM120 under basal growth condition (left), but resistant to this drug under E2-treated condition. D) AKT1KD cells were resistant to NVP-BEZ235 under E2-treated condition. E) AKT2 knockdown reduced sensitivity to MK2206 under E2-treated condition. While

the mean ± SE for MK2206 and NVP-BEZ235 are from biological replicates, results for BKM120 and BYL719 are representative data from technical replicates due to experimental variability.

Figure 6: AKT1 and AKT2 knockdown had minimum effect of E2 signaling in BT-474 cells: A) AKT1, AKT1_pS473, AKT2, and AKT2_pS474 levels in cells treated with control luciferase (luci), AKT1 and AKT2 siRNAs for four days. AKT isoform levels were measured in untreated and heregulin (15 minutes) treated cells. AKT1 and AKT2 activities are mutually dependent in BT-474 cells. B) The effect of AKT1 and AKT2 knockdown on basal proliferation of BT-474 cells. C) The effect of AKT1 and AKT2 knockdown on E2-mediated cell proliferation and response to tamoxifen. Assays were performed as in Figure 5A except that tamoxifen concentration was one micromolar. Due to experimental variability, results of two experiments are shown separately (six replicates in each condition). D) The effect of AKT1 and AKT2 isoform knockdown on E2-regulated expression of SIAH2 and SALL4 as measured by qRT-PCR. E) AKT2 knockdown cells were partially resistant to NVP-BEZ235 compared with control cells. AKT1 knockdown cells showed similar trend ($p=0.06$).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AKT2_pS474
(Long exposure
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WT
WT
WT
WT
T323G
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E545K
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βActin
AKT2_pS474
AKT2
βActin
Set 1
Set 2
Set 3
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AKT3
βActin
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AKT2_pS474 (long expo)
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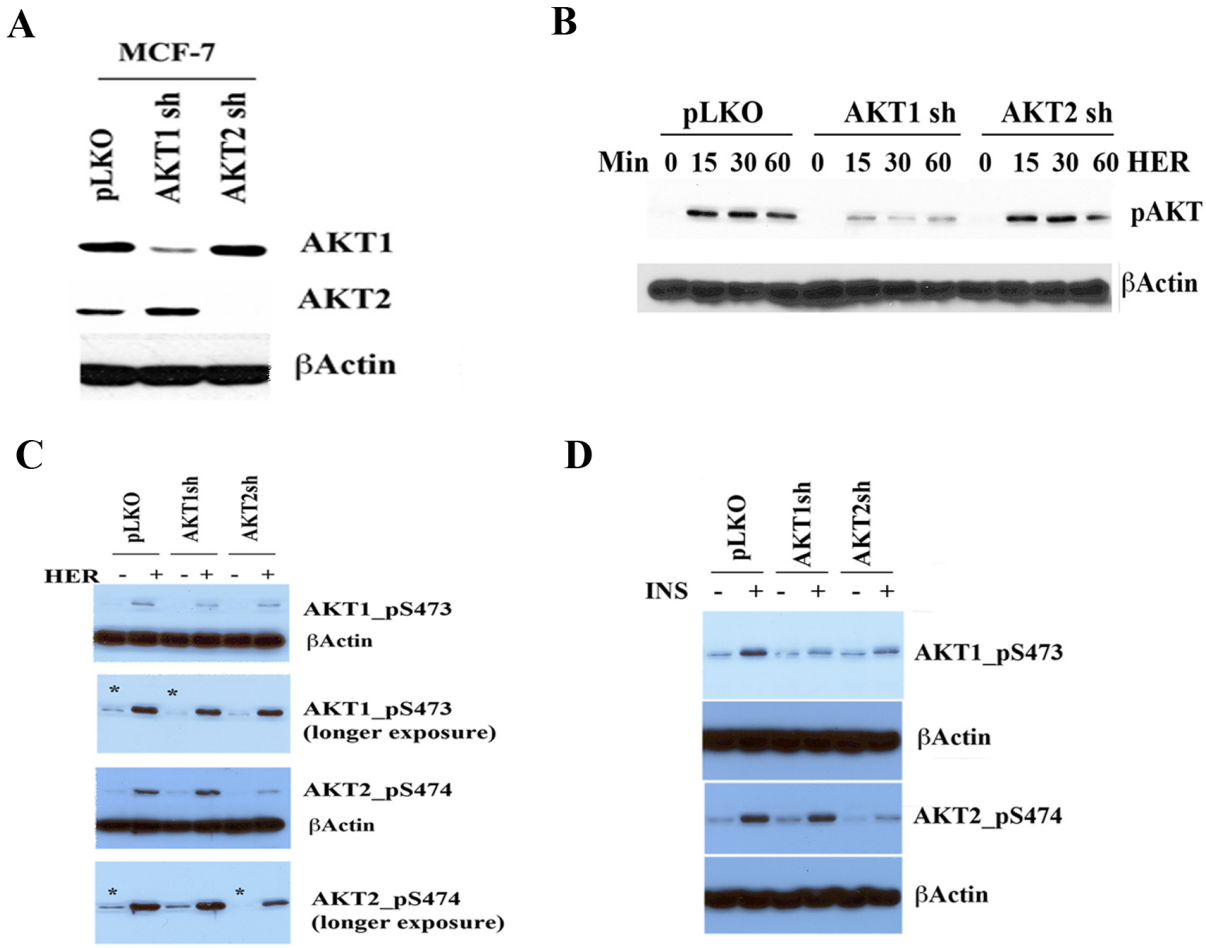
Fig. 1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(longer exposure)
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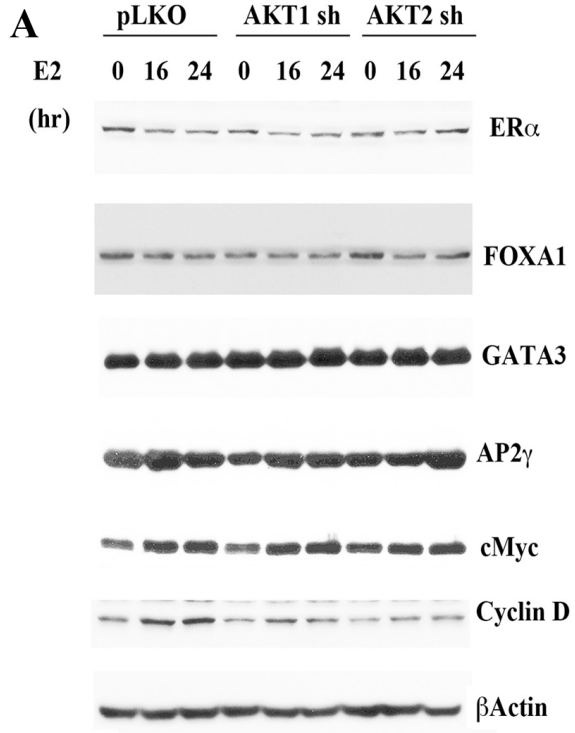
Fig. 2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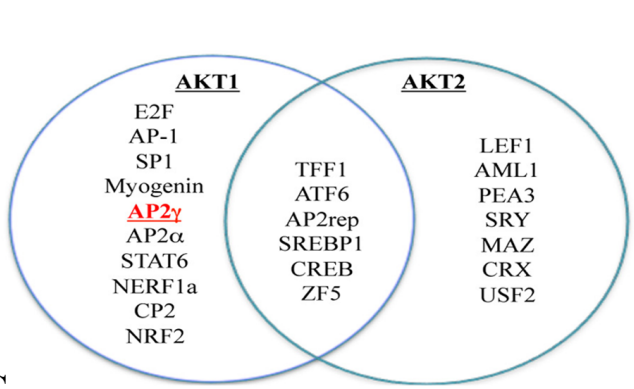
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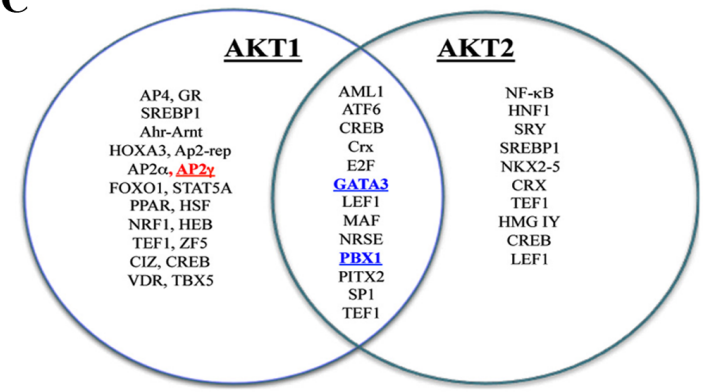
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VDR, TBX5
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CREB
Crx
E2F
GATA3
LEF1
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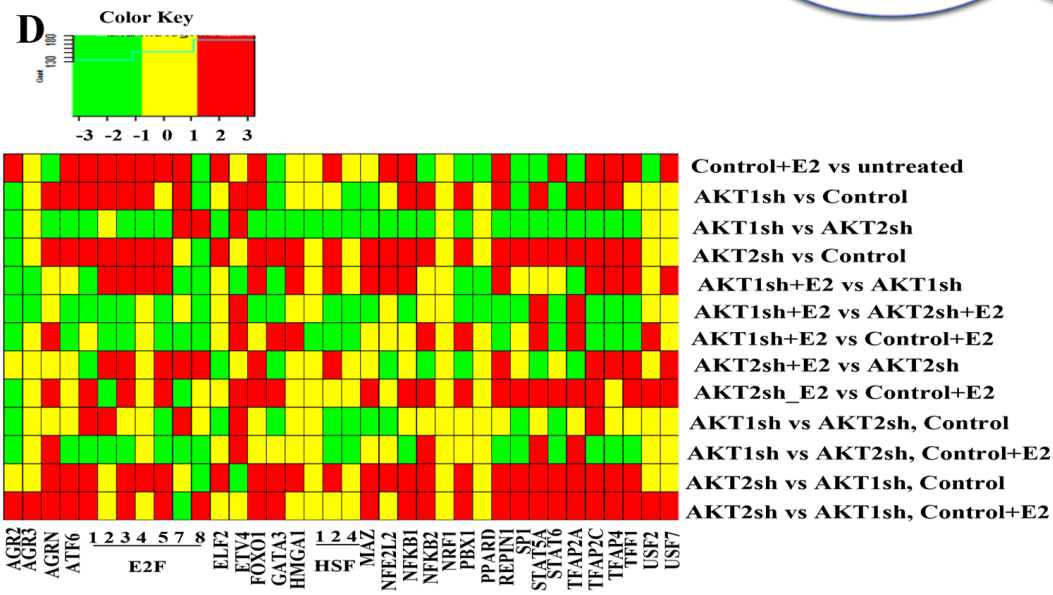
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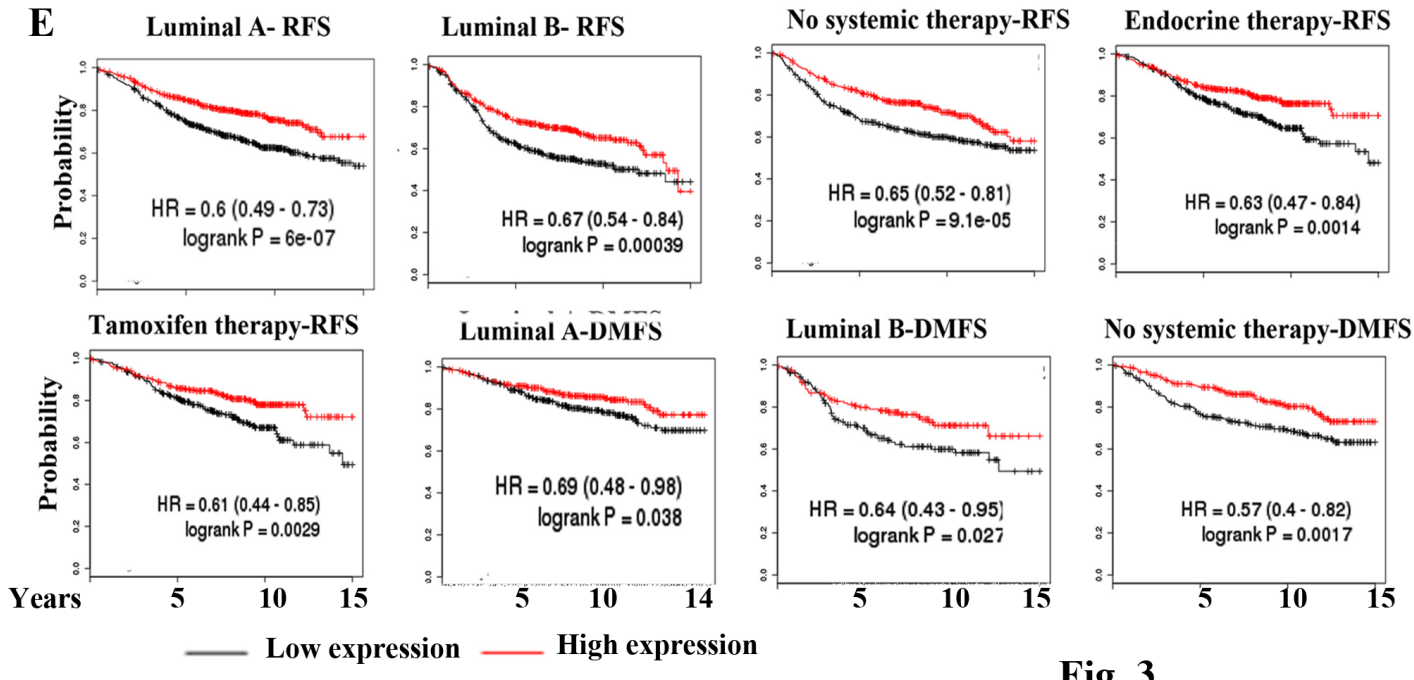
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Luminal A- RFS
Luminal B- RFS
No systemic therapy-RFS
Endocrine therapy-RFS
Probability
HR = 0.6 (0.49 - 0.73)
logrank P = 6e-07
HR = 0.67 (0.54 - 0.84)
logrank P = 0.00039
HR = 0.65 (0.52 - 0.81)
logrank P = 9.1e-05
HR = 0.63 (0.47 - 0.84)
logrank P = 0.0014
Tamoxifen therapy-RFS
Luminal A-DMFS
Luminal B-DMFS
No systemic therapy-DMFS
Probability
HR = 0.61 (0.44 - 0.85)
logrank P = 0.0029
HR = 0.69 (0.48 - 0.98)
logrank P = 0.038
HR = 0.64 (0.43 - 0.95)
logrank P = 0.027
HR = 0.57 (0.4 - 0.82)
logrank P = 0.0017
Years
5 10 15
5 10 14
5 10 15
5 10 15
Low expression
High expression

Fig. 3

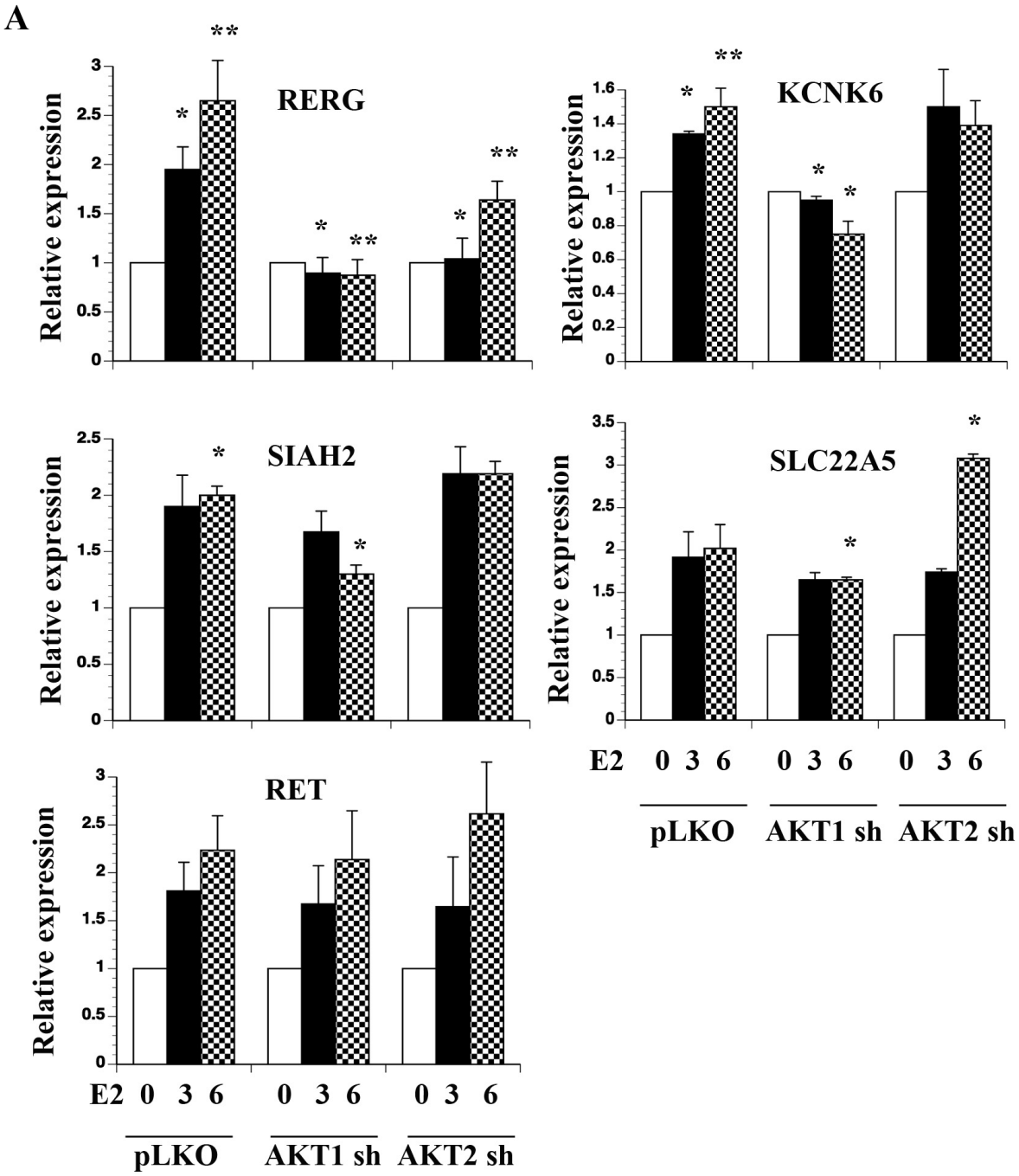
A
RERG
KCNK6
SIAH2
SLC22A5
RET
Relative expression
E2 0 3 6 0 3 6 0 3 6
pLKO
AKT1 sh
AKT2 sh
B
SALL4
BTG2
Relative expression
E2 0 3 6 0 3 6 0 3 6
pLKO
AKT1 sh
AKT2 sh

Fig. 4

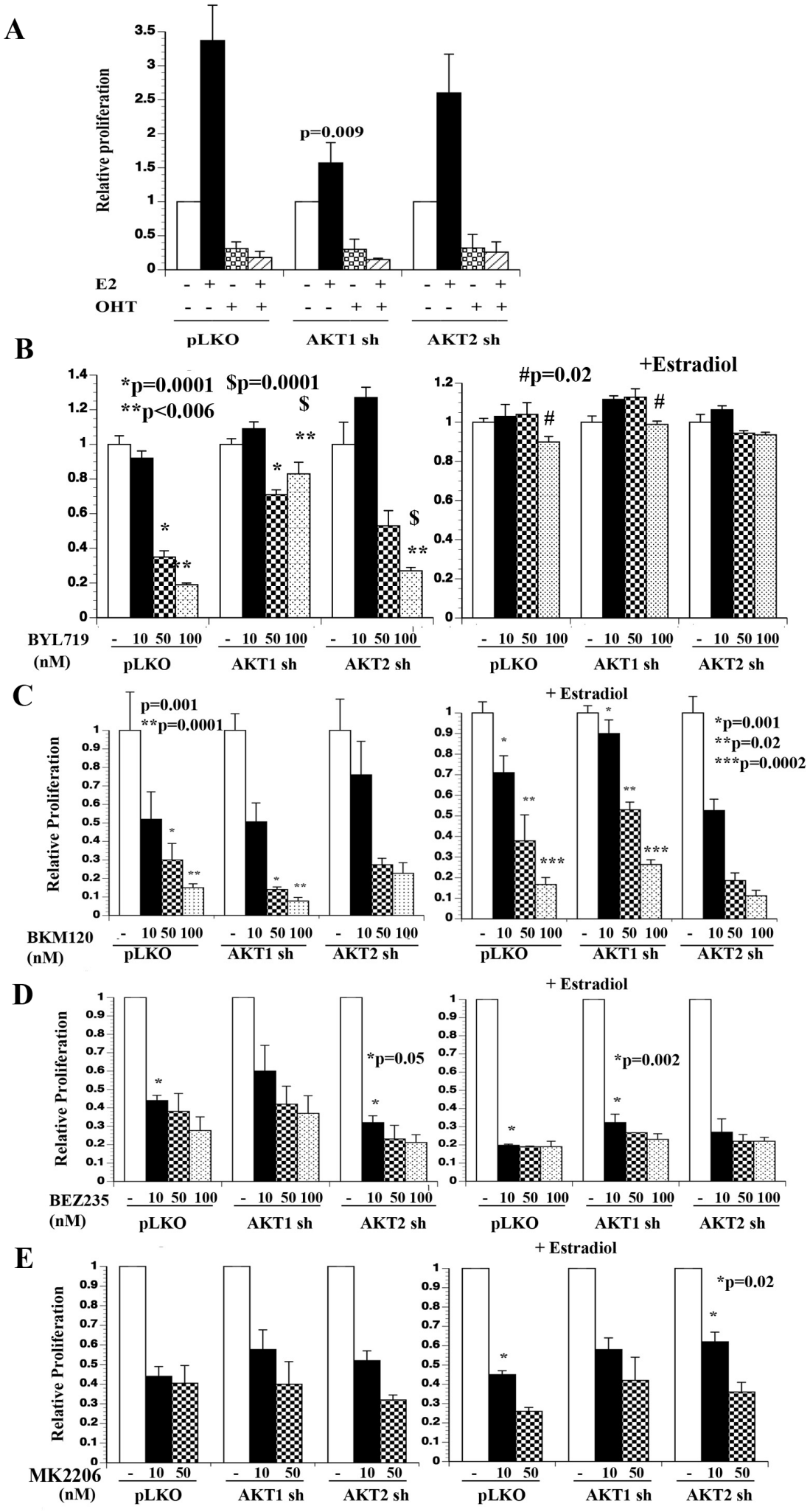
A
Relative proliferation
p=0.009
E2
OHT
pLKO
AKT1 sh
AKT2 sh
B
*p=0.0001
**p<0.006
$p=0.0001
#p=0.02
+Estradiol
BYL719 (nM)
C
p=0.001
**p=0.0001
+ Estradiol
*p=0.001
**p=0.02
***p=0.0002
Relative Proliferation
BKM120 (nM)
D
*p=0.05
*p=0.002
BEZ235 (nM)
E
*p=0.02
MK2206 (nM)

Fig. 5

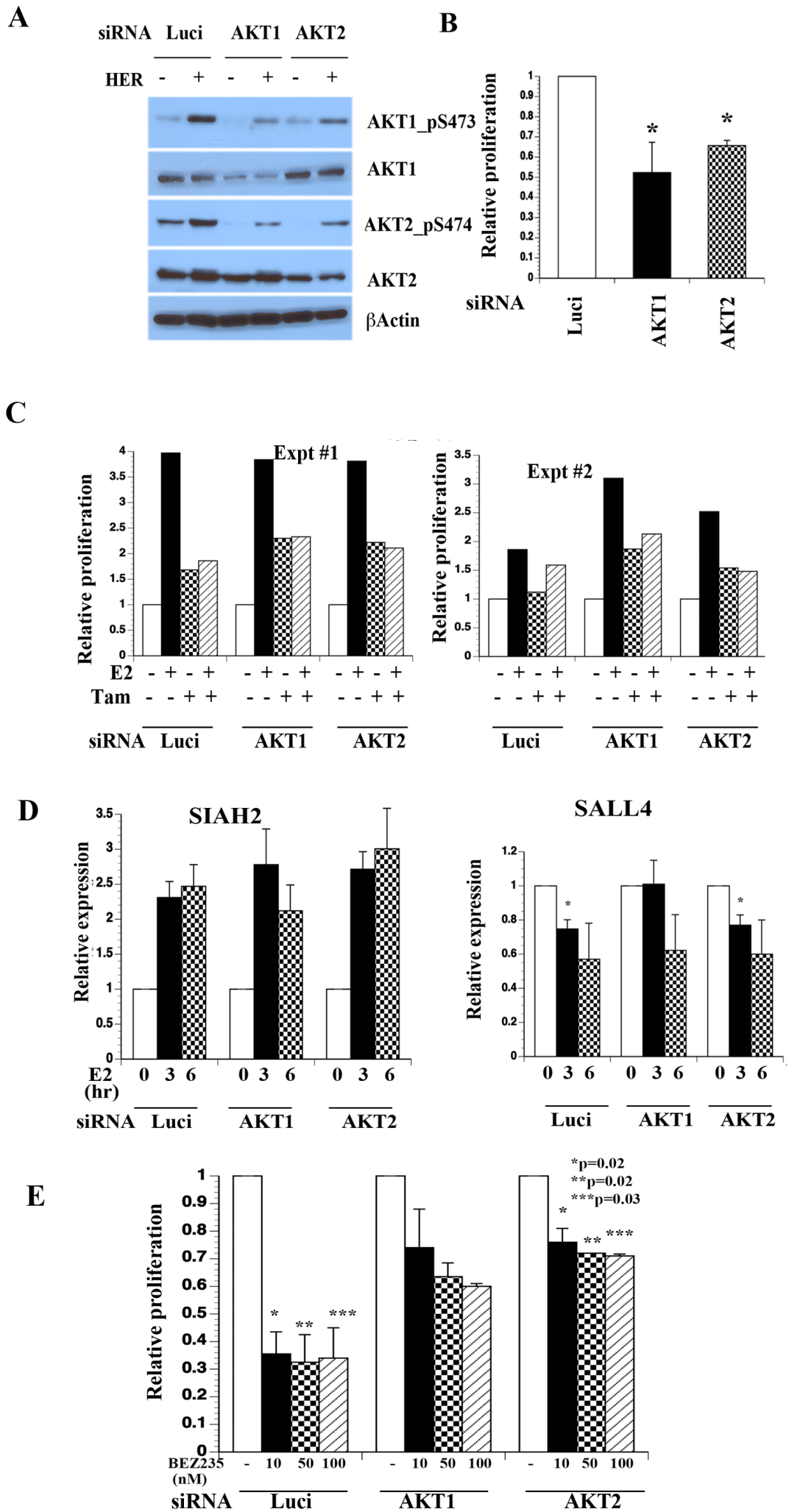
A
siRNA Luci AKT1 AKT2
HER - + - + - +
AKT1_pS473
AKT1
AKT2_pS474
AKT2
βActin
B
Relative proliferation
siRNA
Luci
AKT1
AKT2
C
Expt #1
Expt #2
Relative proliferation
E2 - + - + - + - + - + - + - + - + - + - + - +
Tam - - + + - - + + - - + + - - + + - - + + - -
siRNA Luci AKT1 AKT2
Luci AKT1 AKT2
D
SIAH2
SALL4
Relative expression
E2 (hr) 0 3 6 0 3 6 0 3 6
siRNA Luci AKT1 AKT2
Luci AKT1 AKT2
E
Relative proliferation
*p=0.02
**p=0.02
***p=0.03
BEZ235 (nM) - 10 50 100 - 10 50 100 - 10 50 100
siRNA Luci AKT1 AKT2

Fig. 6